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(54) Title: IMMUNOASSAY FOR THE DETERMINATION OF ANTI-HIV ANTIBODIES IN HUMAN SAMPLES

(57) Abstract

An immunoassay for anti-HIV antibody in a sample comprising the steps of forming an HIV-antigen$_1$:anti-HIV antibody:HIV-antigen$_2$ immune complex and detecting the complex. The novel feature of the assay is that the ternary complex is formed in the presence of an effective amount of an inhibitor for the interaction between CD4 and an HIV envelope protein.

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IMMUNOASSAY FOR THE DETERMINATION OF ANTI-HIV ANTIBODIES IN HUMAN SAMPLES

Field of the invention

This invention relates to an improvement in assaying for anti-HIV antibodies in human derived specimens. The assay makes use of a format in which an immune complex comprising HIV-antigen$_1$:anti-HIV antibody:HIV-antigen$_2$ is formed, indicating the presence of anti-HIV antibody in the specimen.

In connection with the present patent specification the term "antigen" refers to a molecular entity that can bind to an antibody. The term therefore also encompasses haptens

Background

Due to the impact an HIV-positive result will have on an individual's social life, false positive results are not desirable. Furthermore, currently available tests for the presence of anti-HIV antibody need improvement with respect to sensitivity.

Testing blood or blood products for hepatitis and HIV has become common practice. A positive answer for either of the two infective agents will lead to the blood or blood products being discarded. Both types of testing must be performed on blood intended for medical use. In order to minimize the amount of required testing and labor, a sensitive, reliable hepatitis/HIV-combination test is needed.

The present invention is directed toward increasing the sensitivity and reducing the "background" or non-specific "noise" in testing for anti-HIV antibody by the antigen:antibody:antigen-format. The invention is applicable to combination tests for anti-HIV antibody as one analyte, and particularly in combination with testing for hepatitis surface antigen (HBsAg) and potentially also for hepatitis C antigen and antibody directed thereagainst.

Immunoassay protocols utilizing the f rmati n f an antigen$_1$:antibody:antigen$_2$-complex are well-known in the field. For d tecting anti-HIV antibodies see f r instance EP-A-313,986 (Abbott) and EP-A-397,149 (Wellc me). In order to bind to the same antibody, antigen$_1$ and antigen$_2$ must hav one

epit pe in common. N rmally the antigenic entities used (antigen$_1$ and antigen$_2$) are modified in order to facilitate detection of the complex. The antigens employed may be insoluble, such as in certain heterogeneous and agglutination assays, or labeled (conjugated) with an analytically detectable group that, for instance, may be radioactive, fluorescent, chemiluminescent, bioluminescent, enzymatic etc., or exhibit bioaffinity, such as biotin does towards (strep)avidin or a hapten does towards its homologous antibody. An antigen conjugate is an antigen to which an analytically detectable group has been covalently linked.

The general performance of these commonly known assays comprises the steps of contacting, under conditions allowing immune complex formation, a biological sample suspected of containing the antibody with the appropriate forms of antigens that are homologous to the sample antibody and detecting the complex formed. If the antigenic entities used are unmodified, soluble and multivalent, the formation of a precipitate (insoluble immune complex) is an indication of a positive sample. By having both antigenic entities bound to minute particles that are suspended in the assay medium, agglutination assays may be constructed that function in the same general way as the precipitation assays. Depending on the assay construction, the complex could be determined by the naked eye (agglutination), or by nephelometric methods.

The most popular immunoassays of the format involving antigen$_1$:antibody:antigen$_2$-complexes employ antigens (e.g. antigen$_2$) that are labeled with an analytically detectable group, i.e. the amount of the analytically detectable group incorporated into the complex is taken as a measure of the amount of complex formed which in turn is a measure of the amount of antibody in the sample. In order to ensure that the tested antibody is quantatively sandwiched between the two antigens, the antigenic entities have to be added in excess which has lead to two different types f prot cols - the heterogeneous and homogeneous nes. In the heterogeneous assays the complex is separated from excess labelled antigen before quantitation. In order to facilitate separation, one of

the antigens (e.g. antigen$_1$) is insoluble or becomes insoluble during the assay and the other (e.g. antigen$_2$) is labeled with the analytically detectable group. The homogeneous assays employ labels that change their signal as a consequence of being incorporated into the complex, and consequently no separation is required.

The invention and its preferred modes

It has now been realized that the sensitivity and/or specificity of testing for anti-HIV antibodies by the use of the antigen:antibody:antigen-format can be increased by forming the complex comprising HIV-antigen$_1$:anti-HIV antibody:HIV-antigen$_2$ in the presence of an effective amount of an inhibitor for the interaction between an HIV envelope protein (e.g. gp120 for HIV 1 and gp105 for HIV 2) and the corresponding cell receptor (CD4). The amount and inhibitor is effective in the sense that they will reduce the non-specific binding. This type of inhibition has previously been suggested for the blocking of HIV-infections of CD4 target cells. See for instance De Clercq, E. (6th International Conference on AIDS, 21st June, 1990, San Fransisco). Parish C.R. et al. (J. Immunol. 145(1990)1188-) have examined a number of sulfated polyanions for their ability to block anti-CD4 mAb binding. On CD4 they found a polyanion binding site that was clearly distinct but closely associated with the gp120 binding region of CD4. Generally speaking the known inhibitors are polyanionic, in particular polysulfated or polysulfonated (i.e. exhibiting $-SO_3^-$ groups), polymers optionally containing a plurality of OH-groups. Suitable polymers are preferably soluble in aqueous media. Specific examples are sulfated polysaccharides, such as dextran sulfate, heparin, pentosan sulfate, fucoidan, and the carrageenans, and polyvinyl alcohol sulfate, and polyanethole sulfonate.

Addition of the inhibitor reduces the nonspecific binding thereby increasing the specificity and/or sensitivity. The addition replaces the use of HIV antigen$_1$ and HIV antigen$_2$ that derive from different sources in pur antibody immunoassays of the antigen:antibody:antigen f rmat. Compare EP-A-313,986 (Abbott) and EP-A-397,149 (Wellcome).

For an ptimal assay performance the molecular weight, substitution degree, and concentrati n of inhibitor employed may vary between different assay systems and between different inhibitors. In order to have a good test, normal optimization with respect to these variables shall therefore always take place. For instance if the test simultaneously shall detect HBsAg with good sensitivity it might be necessary not to increase the inhibitor concentration too much. See the experimental part of this specification. For an anti-HIV antibody test alone, the concentration of dextran sulfate is recommended to be within 0.01-0.14%(w/w) or a concentration giving an equivalent or better effect if other CD4-gp120 HIV envelope inhibitors are used. In a combination test, for instance, the simultaneous detection of anti-HIV antibody and an hepatitis antigen such as HBsAg, the upper limit should in many cases be lowered, e.g. down to 0.10 %.

In order to demonstrate the effect in the present invention, at least one of the antigenic preparations used (HIV-antigen$_1$ or HIV-antigen$_2$) should contain HIV antigens originating from an HIV grown in a human derived cell line. The preparation can thus be a non-purified, semi-purified or fully purified HIV lysate that has been rendered insoluble, for instance by physically coating on or covalently binding to one of the solid phases used in connection with heterogeneous immunoassays, or rendered detectable by being covalently linked to any of the above-mentioned analytically detectable groups. In certain instances the preparation may also contain recombinantly or synthetically produced HIV-proteins and peptides, such as described by Essex et al (US-A-4,725,669) and Vahlne et al (US-A-4,812,556 (HIV-2 specific peptide) and EP-A-284,587 (HIV-1 specific peptide)). In order to achieve the benefits of the present invention, the other HIV antigen preparation should contain antigenic/haptenic proteins involved in the binding to the CD4 receptor, i.e. an HIV envelope protein or its antibody-binding fragments with binding ability to the CD4 receptor. HIV-antigen$_1$ and/or HIV-antigen$_2$ that either wholly or partly consist of HIV antigens derived from different sources can be used in the invention.

By "derived from different sources" is m ant that they have been obtained by routes starting with different raw materials, for instance one HIV-antigen may have been isolated from native viral material while the other may have been obtained through a synthetic route or by DNA-recombinant techniques by expressing an HIV-antigen in an appropriate host cell.

In particular the present invention relates to assays in which antibodies directed towards an HIV envelope protein or its antibody-binding fragments are detected, e.g. gp120 (HIV-1) and/or gp105 (HIV-2). The assays of the invention may detect these antibodies specifically or together with other HIV-specific antibodies.

In the most preferred embodiment of the invention one of the HIV-antigenic preparations is insoluble (HIV-antigen$_1$), for instance coated on the plastic surface of a mitrotiter well, while the other (HIV-antigen$_2$) is labelled with the analytically detectable group, for instance biotin. Alternative modes include HIV-antigen that is attached to other solid phases known per se in connection with heterogeneous immunoassays. Thus the solid phase could have different forms such as beads, sheets, pads, wells of microtiter plates etc. The support may be porous or non-porous. It may consist of polystyrene, a polysaccharide, nylon, nitrocellulose, polypropylene etc. The artisan will know the material which is compatible with a certain physical form and assay format. The entities coated on the solid phase may be bound thereto merely by physical absorption or covalent attachment. The linkage between the solid phase and the coated entity shall resist normal washing procedures applicable to heterogeneous immunoassays.

The assay conditions required for the immune reaction leading to the complex containing HIV-antigen$_1$:anti-HIV antibody:HIV-antigen$_2$ are essentially the same as normally applied to immunoassays, i.e. aqueous medium, temperature 0-40^{o}C and pH 3-9. Normally a pH-value within 5-9 is appli d. The exact conditions must b ptimized from case to cas . The immune complex formation may be performed stepwise, f r

instance by first binding the sample antibody to HIV-antigen$_1$ and then binding HIV-antigen$_2$ to the thus formed complex between the sample anti-HIV antibody and HIV-antigen$_1$. The reverse reaction order or simultaneous reaction of HIV-antigen$_1$ and HIV-antigen$_2$ with the anti-HIV antibody is also possible. In the case of HIV-antigen$_1$ being bound to a solid phase, it may be advantageous to have washing steps inserted between the steps related to the antigen-antibody reactions. For good performance of the invention, the step finalizing the formation of the complex (HIV-antigen$_1$:anti-HIV antibody:HIV-antigen$_2$) should be run in the presence of the gp120-CD4 inhibitor.

The analytically detectable group employed may be directly or indirectly measurable. Colored or radioactive groups are examples of the former type. Additional examples of direct labels include fluorescent labels such as fluorescein isothiocyanate (FITC) and related compounds such as coumarin and also labels having delayed fluorescence such as certain lanthanide chelates (Eu^{3+}, Sm^{3+}, Tb^{3+} and Yb^{3+}) enabling time-resolved spectrophotometry for the measurement. Examples of indirectly measurable groups are enzymatic groups comprising an enzyme, a substrate, a cosubstrate, a cofactor etc, but important also are bioaffinity labels such as biotin detectable by (strep)avidin conjugates or by anti-biotin antibody conjugates, and other haptenic or antigenic entities detectable by their homologous antibodies. See above for further analytically detectable groups. In case an indirectly measurable label is employed the HIV-antigen$_1$:anti-HIV antibody:HIV-antigen$_2$-complex formed is contacted with a reagent allowing detection of the label. For instance an enzyme label requires addition of enzyme substrate, and biotin requires (strep)avidin or anti-biotin antibody, and a hapten requires the homologous anti-hapten antibody. Bioaffinity labels, such as biotin, require their counterparts to be labeled with an analytically detectable group.

In one asp ct of the present invention anti-HIV antibody testing is part f an HIV/hepatitis combination test. This type of assay is pref rably heterogeneous employing a solid

phase that exhibits both an HIV-antigen and a hepatitis related immune reactant. Examples of the latter one are antibody directed towards HBsAg or towards hepatitis C antigen when a hepatitis antigen is to be detected, and a hepatitis antigen (e.g. hepatitis C antigen) when a anti-hepatitis antibody is to be detected. The reaction steps are essentially the same as the mode assaying for anti-HIV antibodies alone, with the exception that the solid phase after having been incubated with the sample is incubated with both an HIV-antigen and a labeled immune reactant comprising the same specificity as the hepatitis related immune reactant of the solid phase, preferably a labeled anti-HBsAg antibody and/or labeled anti-hepatitis C antibody and/or labeled hepatitis C antigen.

In case of combination tests it is practical to use the same label for each of the respective analytes. For instance the conjugates between biotin and HIV-antigen, between biotin and anti-HBsAg antibody, and between biotin and hepatitis C antigen are useful in HIV/hepatitis-testing. When biotin or a hapten is used as the label, it is preferably detected by the use of a conjugate consisting of anti-biotin or anti-hapten antibody covalently linked to any of the analytically detectable groups given above (except biotin). However this does not exclude the use of different labels for different analytes in combination tests.

In combination tests using solid phases carrying the different immunological counterparts to the analytes in question, the labeled reagents binding to the respective analyte is preferably used as a mixture, i.e the labeled forms of HIV-antigen, anti-HBsAg antibody and hepatitis C antigen is premixed before being incubated with the analyte that in turn preferably has been bound immunologically to a solid phase in a prior step.

In principle the present invention can be applied to samples from any antibody-containing human derived fluid. The sample may thus be whole blood, serum, plasma, urine, lacrimal fluid, breast milk, cerebrospinal fluid, sputum or saliva etc.

The effect of using an inhibitor for the binding of the HIV envelope protein to the CD4 cell receptor in order to increase the sensitivity and specificity of the immunoassays in question is illustrated in the experimental part below.

EXPERIMENTAL PART

METHODS:

Biotinylations: All biotinylations were performed by standard techniques, previously described by Guesdon et al. (J. Histochemistry 27(1979)p.1131), using biotinyl Nhydroxysuccinimid (BNHS) (Cal-Biochem, San Diego, CA, U.S.A.).

Coating of microtiter plates: Polystyrene microtiter plates were coated with purified HIV antigen and mouse monoclonal antibody to HBsAg by a three step process. First, monoclonal antibody to HBsAg was coated in phosphate buffer, 0.1 M, pH 7.2. After incubating 12-16 hours at 4°C, the coating solution was aspirated off and purified HIV antigen was coated in a carbonate buffer, 0.1 M, pH 9.6. Plates were again incubated for 12-16 hours followed by aspiration. In the final step, plates were blocked to prevent non-specific adsorption of immunoglobulins and other serum proteins. The blocking buffer consisted of 0.1 M Tris, pH 7.4 with 1% bovine serum albumin, 5% sucrose, 0.1% (w/v) Tween 20 (Sigma Chemical Co, St. Louis, Mo, U.S.A.) and preservatives.

Assaying protocol: 100 μl of each sample was pipetted into the wells of the microtiter plate. The plate was covered with a sealer and incubated at 37°C for 60 minutes. The plate was aspirated and washed five times with a wash buffer using 250-300 μl per well. 100 μl of the biotinylated reagents (Conjugate A) was added to each well of the microassay plate and a new plate sealer applied. The plate was then incubated at 37°C for 60 minutes. Again the plate was aspirated and washed five times as above. 100 μl goat anti-biotin conjugated to HRP (C njugate B) was added t each well and a new plate sealer was applied. The plate was again incubated at 37°C for 60 minutes and aspirated and washed five times. 100 μl of substrate (OPD in excess) was added to each w ll and the

plates were incubated at room temperatur in the dark for 30 minutes. 100 µl of stop s lution containing 2 N sulfuric acid was added and the developed color (absorbance) read at 492 nm within 60 minutes using a reference wavelength of 600-620 nm.

<u>REAGENTS</u>:

<u>Anti HBsAg antibodies</u>:

Monoclonal antibody to HBsAg was obtained from Sorin (Italy).

Polyclonal antibody to HBsAg was obtained from hyperimmunized goats purified by standard techniques and biotinylated.

<u>HIV antigens</u>:

The HIV antigen used for coating of microtiter plates (HIV antigen$_1$) was derived from HIV-1 infected H9 cell lysate by ultracentrifugation. For use in labeled form (antigen$_2$) the HIV-1 purified lysate was further purified prior to biotinylation by passage through a Sepharose anti-H9 affinity column. Unbound HIV-1 antigen was collected and biotinylated for use in Conjugate A above. The final product demonstrated a higher specific HIV-1 activity, relative to total protein, than prior to affinity purification. The Sepharose anti-H9 affinity column was prepared by covalently attaching goat antibody directed against human H-9 cellular proteins to CNBr activated Sepharose (Pharmacia AB, Uppsala, Sweden). The antibody was prepared by immunizing goats with an immunogen derived from uninfected human H-9 cells. The goats developed an antibody titer to uninfected H-9 cellular proteins, as well as to components that may remain from the H-9 cell culture media. The HIV antigen thus obtained was almost completely lacking H-9 proteins, and as an unavoidable consequence it had also been depleted of gp41, gp120, and gp160 envelope protein determinants

The HIV antigen used as the labeled antigen (HIV-antigen$_2$) was a mixture of biotinylated HIV antigen from H9 cell lysate and biotinylated recombinant HIV gp160 envelope antigen prepared in insect cells (Repligen Corp., Cambridge, Mass., U.S.A.). T

The biotinylated antibody to HBsAg and the biotinylated HIV antigen were used as a mixture (Conjugate A) dissolved in an aqueous medium containing 0.1 M TRIS, 0.15 M sodium chloride, 0.025% Tween 20 (SIGMA, St. Louis, Mo, U.S.A.), 0.04% dextran sulfate, 5% bovine serum albumin (BSA), 0.5% 4-dimethylaminoantipyrin (DAP) as an antioxidant, 20% normal goat serum and 10% normal human serum.

Anti-biotin antibody:

Goat anti-biotin antibody labeled with horseradish peroxidase (Conjugate B) was purchased from Zymed Corp., South San Fransisco, CA., U.S.A. The antibody was used in a Tris buffered diluent of the same composition as above.

HRP substrate: ortho-phenylenediamine (OPD) dissolved in a buffer containing potassium phosphate, sodium citrate and hydrogen peroxide, pH 5.0.

Wash Buffer: Saline buffered with sodium phosphate and containing a surfactant.

Samples: See under results.

Dextran sulfate: Purchased from Sigma Co., St. Louis, Mo., U.S.A.

RESULTS:

See the table in which absorbance values are given.

	Dextransulfate % (w/w)				Heparin%(w/w)
Samples	0.00	0.16	0.08	0.04	0.04
A	0.056	0.047	0.047	0.049	0.060
B	0.055	0.048	0.045	0.049	0.057
C	0.137	0.072	0.068	0.087	0.086
D	0.071	0.057	0.065	0.059	0.071
E	0.242	0.183	0.199	0.238	0.249
F	1.168	0.599	1.028	1.029	1.098
G	1.748	1.308	1.700	1.947	1.851
H	3.158	3.135	3.183	3.164	3.194

A representative panel of samples (A-H) were examined. The cut off value for the assay has preliminarily been fixed to an absorbance of 0.110.

A. A normal human serum pool (NHS). Absorbances should be as low as possible.

B. A normal human plasma pool (NHP). Absorbances should be as low as possible.

C. CPDA 3654-20A: A human citrated plasma that is negative for anti-HIV antibody and HBsAg, but consistently has given elevated absorbances in a HIV/HBsAg combination test without addition of heparin or dextran sulfate. Without an inhibitor added this sample would appear as a false positive. A pronounced effect on increasing the non-specific (background) absorbance value is observed.

D. Travenol H94644: A sample that is similar to CPDA 3654-20A and frequently causes elevated absorbances. The sample is not truely positive for anti-HIV antibody or HBsAg. The test will classify this sample as a true negative. The effect of increasing the background is less pronounced than for the previous samples.

E. Ay 0.156 ng/mL: Paul Ehrlich Institute HBsAg standard containing 0.156 ng/mL antigen. The effect of dextran sulfate is considerable. However, the sample is negative for anti-HIV antibody. The results indicate that in combination tests involving HBsAg too high a dextran sulfate concentration has a negative influence on the HBsAg sensitivity.

F. Low p24 E1: A true positive human serum sample that contains antibodies to all HIV proteins except the p24 core protein. The sample has been included as a panel member because an anti-HIV antibody test that relies heavily on anti-p24 antibodies for optimal performance would perform poorly with this sample. The result indicates that low anti-p24 antibody sample should not be assayed in the presence of too high a dextran sulfate concentration (>0.08% w/w), assay sensitivity can be reduced.

G. 1:100 (MPC) #6709200: An anti-HIV antibody positive human plasma that is diluted 1:100 in n gative human plasma. MPC stands for Medium Positive Control. All absorbances are

similar for all concentrations, except that 0.16 % concentration of dextran sulfate apears to have a negative impact on the sensitivity.

H. D1: A strong anti-HIV antibody positive sample. No impact was noted on the absorbances values for any inhibitor concentration.

In conclusion the data support that the invention provides a reduction of "noise" or " background" shown by reduced absorbances with samples that do not contain the analyte of interest. The net effect is better resolution and discrimination between a truly "negative" sample and a low level positive, resulting in improved sensitivity. Improved specificity (reduced number of false positives) is likewise obtained by a reduction in absorbance below the cut-off level for certain samples that do not contain antibody or antigen.

PATENT CLAIMS

1. In an immunoassay for anti-HIV antibody in a human derived biological sample comprising the steps of forming a complex containing HIV-antigen bound to sample anti-HIV antibody bound to HIV-antigen (HIV-antigen$_1$:anti-HIV antibody:HIV-antigen$_2$) and detecting the complex, whereupon the presence of said complex is taken as an indication of the sample being derived from an HIV infected individual,
the improvement being that the formation of said complex is performed in aqueous medium in the presence of a non-specific binding reducing effective amount of an inhibitor for the interaction between CD4 and an HIV envelope protein.

2. An immunoassay according to claim 1, wherein the inhibitor is dextran sulfate.

3. An immunoassay according to claim 1 being heterogeneous in the sense that HIV-antigen$_1$ is insoluble in the reaction medium and the HIV-antigen$_2$ is labeled with an analytically detectable group.

4. An immunoassay according to claim 3,wherein the inhibitor is dextran sulfate.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07976

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(5): G01N 33/536
U.S.: 435/7.1

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/5, 7.1, 7.72, 7.8, 7.9, 7.92-94

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	THE JOURNAL OF IMMUNOLOGY, Volume 145, issued 15 August 1990, Parish et al, "A Polyanion Binding Site on the CD4 Molecule, Proximity to the HIV-gp120 Binding Region", pages 1188-1195, see entire document.	1-4

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 January 1992	10 FEB 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Jeffrey Stucker

Form PCT/ISA/210 (second sheet) (Rev.11-87)